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REMARKS/ARGUMENTS

Claims 11-13 are pending in this application.

Claim 11 recites that “the antibody has a positive reactivity against myeloid cells.”

The fact that the antibody of the present invention has a positive reactivity to myeloid cells is shown in Table 1 in the specification of this application (see column of RS38, in the column of Myeloid in Table 1 of the specification). Claims 12 and 13 correspond to Claims 9 and 10, respectively.

The rejection of Claims 8 and 9 under 35 U.S.C. 102(a) over Goto et al. 1992 in view of WO 98/35698 is believed to be obviated by the amendment submitted above. Goto et al. do not disclose the antibody recited in Claim 11.

Goto et al. do not disclose whether or not the HM1.24 antibody has a positive reactivity against myeloid cells. However, an article published by the same authors, Goto et al., Blood, Vol. 84, No. 6, 1992-1993 (1994) (Goto et al. 1994), a copy of which is submitted herewith, discloses the properties of the HM1.24 antibody, which is same antibody described in Goto et al. 1992.

In particular, Goto et al. 1994 discloses that the HM1.24 antibody does not exhibit any reactivity against myeloid cells, such as acute myeloblastic leukemia and chronic myelogenous leukemia and so on (see reactivity of Anti-HM1.24 in Table 1 at page 1924 of the reference).

Accordingly, Goto et al. 1992 fails to disclose the claimed antibody. Withdrawal of this ground of rejection is respectfully requested. I

Application No. 09/828,217  
Reply to Office Action of May 29, 2003

Applicant submits the present application is in condition of allowance. Early notice to this effect is earnestly solicited.

Respectfully submitted,

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## A Novel Membrane Antigen Selectively Expressed on Terminally Differentiated Human B Cells

By Tetsuya Goto, Stephen J. Kennel, Masahiro Abe, Makoto Takishita, Masaaki Kosaka, Alan Solomon, and Shiro Saito

A monoclonal antibody (MoAb) that defines a novel terminal B-cell-restricted antigen, termed HM1.24, was developed against a human plasma cell line. The MoAb, designated anti-HM1.24, reacted with five different human myeloma cell lines, as well as with monoclonal neoplastic plasma cells obtained from the bone marrow or peripheral blood of patients with multiple myeloma or Waldenström's macroglobulinemia. The HM1.24 antigen was also expressed by mature Ig-secreting B cells (plasma cells and lymphoplasmacytoid cells) but not by other cells contained in the peripheral blood, bone marrow, liver, spleen, kidney, or heart of normal individuals or patients with non-plasma-cell-related malignancies.

The anti-HM1.24 MoAb bound to human myeloma RPMI 8226 cells with an affinity constant of  $9.2 \times 10^6 \text{ M}^{-1}$ , indicating ~84,000 sites/cell. By immunoprecipitation assay under reducing conditions, this MoAb identified a membrane glycoprotein that had a molecular weight of 29 to 33 kD. Our studies indicate that the HM1.24-related protein represents a specific marker of late-stage B-cell maturation and potentially serves as a target antigen for the immunotherapy of multiple myeloma and related plasma cell dyscrasias.

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**H**UMAN B CELLS mature into antibody-producing plasma cells through a number of stages defined by distinctive phenotypic markers. The terminal stage of B-cell differentiation is recognized by the acquisition of cytoplasmic Ig (cIg) and the loss of such common B-cell antigens as surface Ig, HLA-DR, CD20, and Fc receptors and C<sub>3</sub> receptors.<sup>1</sup>

Although several monoclonal antibodies (MoAbs), including anti-PCA-1,<sup>2</sup> anti-PC-1,<sup>3</sup> and MM4,<sup>4</sup> have been reported to detect plasma-cell-associated membrane antigens, anti-CD38 MoAbs were still used for the detection of plasma cells and myeloma cells even in recent studies,<sup>5-11</sup> because of their consistent and strong reactivity. The anti-CD38 MoAbs might be useful tools for the immunophenotyping of plasma cells, providing that multiparameter flow cytometric analyses were used to ensure the precise targeting.<sup>5A,10</sup> However, CD38 is an activation-associated, rather than differentiation-associated, antigen, lacking lineage restrictions,<sup>12</sup> found on a variety of cells.<sup>13-16</sup> Moreover, this antigen is strongly expressed on lineage-committed hematopoietic progenitor cells,<sup>17</sup> whereas it is not present on certain lymphoplasmacytoid tumor cells.<sup>1</sup> For these reasons, CD38 may not be a suitable target antigen for the study of human B-cell maturation and immunotherapy of plasma cell dyscrasias.

To identify a specific marker of terminally differentiated B cells, we prepared a series of MoAbs against a plasma cell line, designated KPC-32. The present report describes the characterization of one such MoAb that identifies a novel membrane antigen, HM1.24, selectively expressed on terminally differentiated normal and neoplastic B cells.

### MATERIALS AND METHODS

**Human cell lines.** Epstein Barr virus-nuclear antigen (EBNA)-negative plasma cell lines KPC-32 and MHB, and a bile duct adenocarcinoma line GBC-YO were established in our laboratory. The KPC-32 cell line was originated from the bone marrow of a male Japanese patient with multiple myeloma.<sup>18</sup> The cells had typical plasmacytoid morphology and produced cytoplasmic  $\lambda$  light chains. Interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-2, IL-6, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interferon- $\gamma$  (IFN- $\gamma$ ) were not detectable in the culture supernatant of the cells by enzyme-linked immunosorbent assay (ELISA) and their growth was independent of IL-6. Plasma-cell line K737<sup>19</sup> was kindly provided by Dr Carmen B. Lozzio (University of Tennessee Medical Center, Knoxville, TN). The following cell lines were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan): plasma cells RPMI 8226, lymphoblastoid cells IM-9 and HS-Sultan (derived from patients with myeloma), Raji and Daudi (Burkitt's lymphoma), CEM and Molt-4 (T-cell leukemia), KG-1 (acute myeloblastic leukemia), KU812F (chronic myelogenous leukemia), K562 (erythroleukemia), THP-1 (monocytic leukemia), U937 (histiocytic lymphoma), WDr (colon adenocarcinoma), Lu-134-A-H (small cell lung carcinoma), and A-172 (glioblastoma). U266 (plasma cell) and MC116 (B-cell lymphoma) cells were obtained from the American Type Culture Collection (Rockville, MD). These cell lines were maintained in culture containing a 1:1 mixture of Ilum's F-12 medium and Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS; Whittaker Bioproducts, Inc, Walkersville, MA), insulin (2 mg/L), transferrin (2 mg/L), ethanolamine (0.122 mg/L), and sodium selenite (9.14 mg/L).

**Human specimens.** Peripheral blood mononuclear cells (PBMC) and nucleated bone marrow cells were obtained by Ficoll-Paque (Pharmacia LKB Biotechnology, Uppsala, Sweden) density gradient centrifugation from healthy volunteers and used immediately. The cell samples from the patients were cryopreserved in -196°C vapor-phase liquid nitrogen with FCS containing 10% dimethylsulfoxide. Biopsy/autopsy tissues were kept at -70°C as snap-frozen blocks and used to prepare sections at the time of experiments. The frozen sections were fixed with acetone for 10 minutes at room temperature and air-dried thoroughly before staining. All procedures involving human specimens were performed according to the protocol approved by the institutional review board for human protection.

**Preparation of MoAbs.** Specific pathogen-free BALB/c mice

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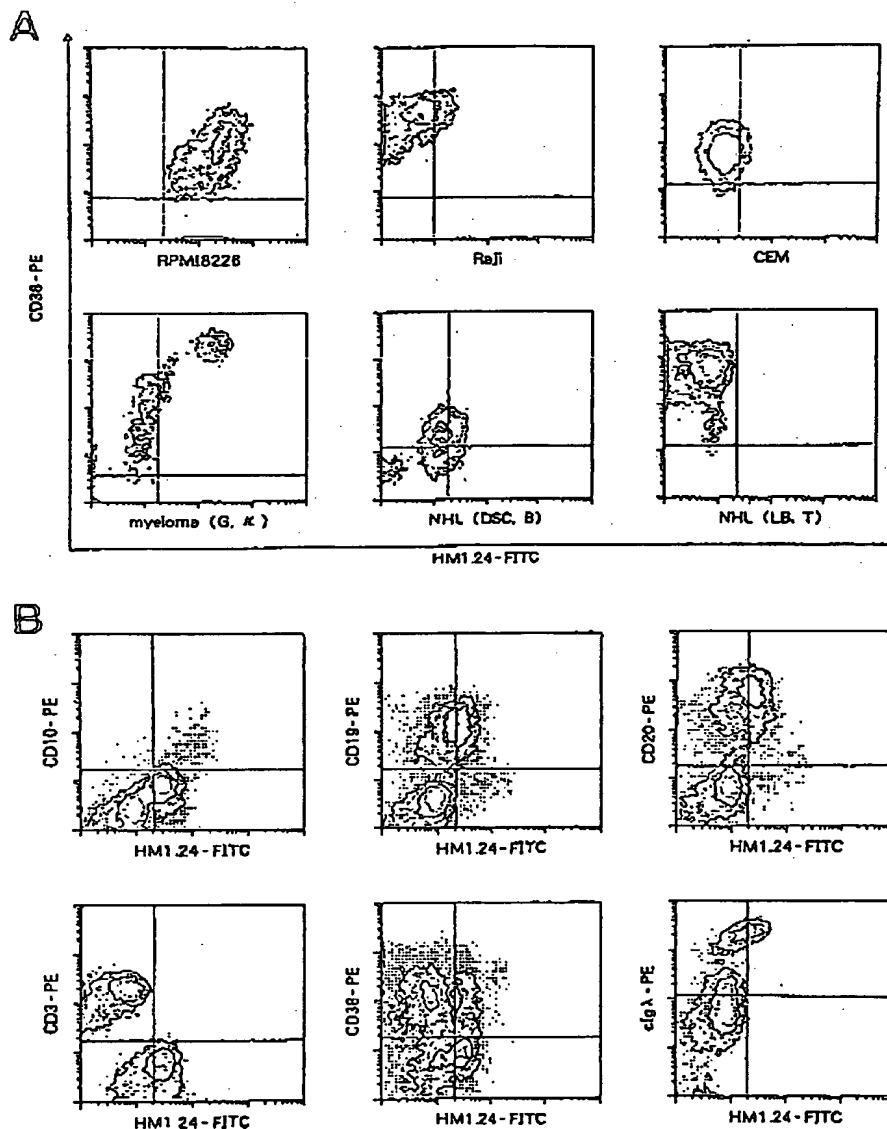
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**Fig 1.** Two-color flow cytometric analysis. Samples were stained directly with FITC-conjugated anti-HM1.24 in combination with PE-conjugated MoAbs to other surface markers. To determine negative regions, isotype-matched controls from the same sources or opposite-light chain controls were always run for each sample. Overlap of emission spectra was compensated for electronically with use of appropriate control samples. (A) Expression of HM1.24 and CD38 on human cell lines and corresponding lymphoid malignancies (NHL, non-Hodgkin's lymphoma; DSC, diffuse small-cleaved cell; LB, lymphoblastic lymphoma). (B) Coexpression pattern of HM1.24 and other differentiation markers on circulating tumor cells from a patient with Waldenström's macroglobulinemia. To identify coexpression of HM1.24 antigen and cytoplasmic light chain, cells were reacted with FITC-anti-HM1.24 in PBS/GS without  $\gamma$ -globulin, washed with PBS, and fixed with 2% paraformaldehyde in PBS at room temperature for 15 minutes. The cells were washed and fixed again with 75% ice-cold ethanol for 5 minutes. After washing, the cells were resuspended in PBS/GS containing 0.25% Tween 20 and stained with affinity-purified PE-Flab<sub>2</sub> fragments of goat antihuman  $\kappa$  or  $\lambda$  antibody (Tago). The fluorescence intensity of FITC-anti-HM1.24 was limited to some extent by the ethanol fixation.



(Charles-River Atsugi, Japan) were immunized with six biweekly intraperitoneal (IP) injection of  $1 \times 10^7$  intact KPC-32-plasma cells. Three days before the cell fusions, selected animals received a final booster of  $1.5 \times 10^6$  KPC-32 cells by intrasplenic injection.<sup>20</sup> Fusions of immune spleen cells and SP2/0 myeloma cells were conducted by the method of de St Groth and Scheidegger.<sup>21</sup> Hybridomas were primarily screened for antibody production by the ELISA using plates coated with intact KPC-32 cells as described below. To exclude hybridomas secreting antibodies against Ig determinants, the positive supernatants were preabsorbed with pooled human serum and screened by ELISA for their reactivity to other cell lines. Selected hybridomas were cloned and tested by flow cytometry against various cell lines and human specimens. Finally, selected clones

were recloned twice and injected intraperitoneally into pristane-primed BALB/c mice and the ascitic fluid harvested.<sup>22</sup> The Ig subclasses of MoAbs were determined by ELISA with subclass-specific rabbit-antimouse antibodies (Zymed, San Francisco, CA). The MoAbs were purified from ascites by ammonium sulfate precipitation<sup>23</sup> and a protein A-affinity chromatography kit (Ampure PA; Amersham Japan, Tokyo, Japan). Purified MoAbs were coupled with fluorescein isothiocyanate (FITC) using a Quick Tag FITC conjugation kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions.

**ELISA.** An ELISA technique using intact target cells described by Posner et al<sup>24</sup> was used for screening antibody activity in hybridoma culture supernatants. Briefly  $5 \times 10^4$  target cells in 50 mL of

Table 1 Reactivity of Anti-HM1.24 Against Malignant Hematopoietic Cells

Diagnosis	No. of Samples	Reactivity of Tumor Cells*	
		Anti-HM1.24	Anti-CD38
Multiple myeloma	12	++	++
Plasma cell leukemia	2	++	++
Macroglobulinemia	2	+	+
B-cell non-Hodgkin's lymphoma	5	±	+
B-cell chronic lymphocytic leukemia	5	0	0 ~ ±
CD19 <sup>+</sup> acute lymphoblastic leukemia	2	0	+ ~ ++
T-cell lymphoblastic lymphoma	2	0	++
Acute myeloblastic leukemia	5	0	+
Chronic myelogenous leukemia	2	0	ND

\* As determined by flow cytometry: 0, negative; ±, weak (NHL in Fig 1); +, moderate (macroglobulinemia in Fig 2); ++, strong (myeloma in Fig 1); ND, not determined.

phosphate-buffered saline (PBS) were dispensed to each well of 96-well plates (U-bottomed; Corning, Iwaki Glass Co, Tokyo, Japan). After blocking with 1% bovine serum albumin (BSA)/PBS, incubation with supernatants was performed at 4°C for 2 hours, followed by a peroxidase-labeled goat antimouse IgG antibody (Zymed) at 4°C for 1 hour and o-phenylenediamine substrate solution (Sumitomo Bakelite, Tokyo, Japan) at room temperature for 30 minutes with washing in between. Color development was terminated by addition of 2 N sulfuric acid and the reaction measured at 492 nm using an ELISA reader (Bio-Rad, Richmond, CA).

**Flow cytometry and cell sorting.** Hybridomas were initially screened by an indirect immunofluorescence technique. Aliquots of  $1 \times 10^6$  target cells were resuspended in 0.5 mL of PBS containing 2% normal goat serum, 0.02% sodium azide (PBS/GS), and 7.5 mg/mL of human  $\gamma$ -globulin (Green Cross Co, Osaka, Japan) to block Fc receptors. After 15 minutes, each cell sample was incubated with 0.5 mL of hybridoma supernatant at 4°C for 1 hour. The cells were washed with PBS/GS and stained with FITC-conjugated F(ab')<sub>2</sub> fragments of goat antimouse IgG(H+L) antibody (Tago Inc, Burlingame, CA) at 4°C for 30 minutes. After washing, the cells were analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). For direct fluorescent staining, we prepared FITC-conjugated anti-HM1.24 (the molar ratio of fluorescein to protein was 1.32) and purchased phycoerythrin (PE)-conjugated MoAbs CD3 (anti-Leu-4), CD19 (anti-Leu-12), CD20 (anti-Leu-16), and CD38 (anti-Leu-17) from Becton Dickinson; and PE-conjugated MoAbs CD10 (anti-J5) and CD21 (anti-B2) from Coulter Diagnostics (Hialeah, FL). In sorting experiments, nucleated bone marrow cells were directly stained with FITC-anti-HM1.24 (without the  $\gamma$ -globulin blocking), the positive cells were sorted on EPICS-CS cell sorter (Coulter), and cytospin slides of each sort were prepared. The slides were stained with Wright-Giemsa stain or fixed with acetone and stained for cytoplasmic light chains with biotinylated MoAbs to human  $\kappa$ - or  $\lambda$ -chains made in our laboratory.<sup>24</sup> The bound MoAbs were detected by ABC-peroxidase reagents (Vector Laboratories, Burlingame, CA). Five hundred cells were examined for the morphology and the presence of clg in each slide.

**Immunocytochemistry and immunohistochemistry.** The immunoperoxidase or alkaline phosphatase techniques (Vectastain ABC kit; Vector) used to examine the reactivity of anti-HM1.24 with cells in the cytospin or tissue specimens were described previously.<sup>25</sup>

**Pokeweed mitogen (PWM) treatment of PBMC *in vitro*.** PBMC from healthy volunteers ( $n = 3$ ) were cultured in the presence of 5  $\mu$ g/mL PWM (Sigma Chemical, St Louis, MO) at  $1 \times 10^6$  cells/mL

and harvested on days 0, 3, 5, and 10. Expression of HM1.24 antigen and other surface markers on the stimulated PBMC were analyzed by two-color flow cytometry.

**Direct MoAb binding.** Purified anti-HM1.24 was radioiodinated with <sup>125</sup>I using limiting amount of chloramine T and further purified by gel filtration on Ultrogel ACA 34 (Pharmacia) in the presence of 5 mg/mL BSA.<sup>26</sup> Intact target cells (RPMI 8226, MHB, IM-9, and THP-1;  $1 \times 10^6$  cells in PBS) were exposed to serially diluted [<sup>125</sup>I]-anti-HM1.24 for 1 hour at 4°C. After washing, bound [<sup>125</sup>I]-anti-HM1.24 was quantitated in a gamma scintillation counter (Beckman, Brea, CA). The association constant of anti-HM1.24 was determined by Scatchard plots using nonlinear regression analysis. For specificity testing, the binding of [<sup>125</sup>I]-anti-HM1.24 ( $1 \mu$ g =  $5.35 \times 10^6$  cpm) was competed for RPMI 8226 cells with a 2,500-fold excess of unlabeled MoAbs anti-HM1.24, anti-CD38 (OKT-10; Ortho Diagnostic Systems, Raritan, NJ), anti-PCA-1 (Coulter), or MM4 (kindly furnished by Dr Alex W. Tong, Baylor University Medical Center Dallas, TX).

**Immunoprecipitation.** RPMI 8226 cells were surface-labeled with carrier-free <sup>125</sup>I using lactoperoxidase as described previously.<sup>27</sup> After labeling, the cells were washed and solubilized by sonication in lysis buffer (PBS containing 5 mg/mL BSA, 0.5% Nonidet P-40, 1 mmol/L sodium azide, and 0.005% phenylmethylsulfonyl fluoride). The cell-lysate was clarified by centrifugation for 1 hour at 100,000g, precleared with normal mouse IgG and antimouse secondary antibody (Cappel-Organon Teknica Co, West Chester, PA), and served as the substrate for the specific immunoprecipitation with the anti-HM1.24 MoAb. Immunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and autoradiography were performed as described previously.<sup>28</sup>

## RESULTS

**Production of MoAbs.** KPC-32 cells were used for the immunization, because these cells lacked known surface markers, including surface Igs, CD3, 10, 11b, 13, 14, 15, 19, 20, 21, 22, 24, 33, 41a, 56, and HLA-DR, except for CD38 and CD71. After fusions with spleen cells from the immunized mice, 141 of 2,304 cultures screened were found to react with the immunizing cells but not with any Ig-related determinants. Subsequent screening by ELISA showed that 30 cultures contained MoAbs reactive with both KPC-32 and RPMI 8226, but not with Raji, CEM, and THP-1. After cloning, supernatants from these cultures were tested against other cell lines and normal PBMC. Most of the 30 clones produced MoAb cross-reacting with non-B-cell lineage and/or PBMC or with cytoplasmic determinants present in acetone-fixed cells. Only three clones were found to produce plasma-cell-specific MoAbs. Because an MoAb of IgG<sub>2a</sub>- $\kappa$ , designated anti-HM1.24, was most useful in flow cytometric analysis and had complement-mediated cytolytic activity against RPMI 8226 cells, this MoAb was selected for further characterization.

**Specificity of anti-HM1.24 on cell lines.** Twenty-one human cell lines, including 5 plasma cell lines, representing various stages of hematopoietic differentiation or nonhematopoietic organs, were analyzed to evaluate specificity of anti-HM1.24 MoAb by direct immunofluorescence. The reaction of this MoAb was exclusively against B cells and, in particular, with plasma cells (5 of 5 positive). Two Epstein-Barr virus-positive (EBV<sup>+</sup>) lymphoblastoid myeloma-related B-cell lines, IM-9 and HS-Sultan, showed higher levels of reactivity than did other B-cell lymphoma-derived lines. Unlike anti-CD38 anti-HM1.24 was unreactive with non-

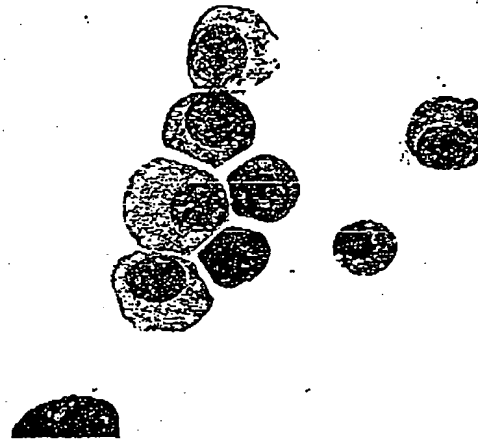
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A



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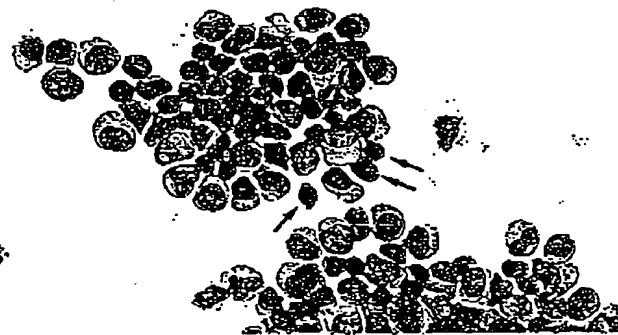


Fig 2. Morphology of HM1.24<sup>+</sup> cells (Wright-Giemsa stains; original magnification,  $\times 1,250$ ). Cells were separated by Epics CS cell sorter from the bone marrow samples of patients with (A) rheumatoid arthritis; (B) IgG $\kappa$  myeloma; and (C) IgG $\lambda$  myeloma. Most cells had a eccentric nucleus, clock-like dispersed chromatin, and basophilic cytoplasm with perinuclear clearing typical for plasma cells. Lymphocytoid cells are indicated by arrows. Immunocytochemical demonstration of cytoplasmic  $\lambda$  light chains in HM1.24<sup>+</sup> cells from the patient in (C): (D) anti- $\kappa$  MoAb; (E) anti- $\lambda$  MoAb; (F) negative control MoAb. (Original magnification  $\times 420$ .)

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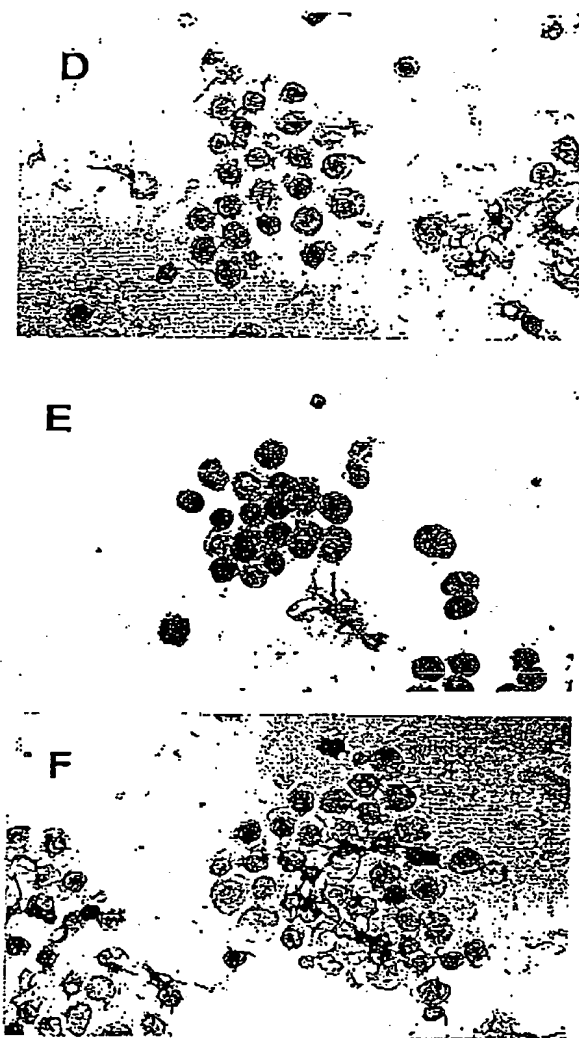


Fig 2. (Cont.d)

B-cell lines and carcinoma lines. The fluorescence-intensity profiles using these reagents are shown in Fig 1A.

**Reactivity of anti-HM1.24 against malignant hematopoietic cells.** As indicated in Table 1, the specificity of anti-HM1.24 MoAb was limited to myeloma and lymphoplasmacytoid cells. Additionally, there were obvious differences in the distribution on the cells of the HM1.24 and CD32 determinants (Fig 1A). Plasma cells were both HM1.24<sup>+</sup> and CD38<sup>+</sup> (Fig 1A, left). Certain phenotypically well-differentiated B-cell populations (obtained by biopsy from patients with malignant lymphoma) exhibited faint staining with the anti-HM1.24, whereas midstage and early stage B cells were unreactive (Table 1). In contrast, anti-CD38 reacted with most of the malignant T and myeloid cells as well as with B-cell malignancies.

Tumor cells of Waldenström's macroglobulinemia may exemplify the B cells at late to terminal phases of differentiation.<sup>29,30</sup> Analyses of such tumor cells ( $n = 2$ ) by flow cytometry involved usage of FITC-anti-HM1.24 in combination with the other anti-B-cell reagents and cIgs. Representative two-color histograms of PBMC from a macroglobulinemia patient who has circulating IgM- $\lambda$ <sup>+</sup> cells are presented in Fig 1B. HM1.24<sup>+</sup> cells did not express CD10 (a marker for pre-B and early stage activated B cells)<sup>31</sup> and CD21 (a marker for midstage B cells<sup>29</sup> [not shown]), but partially coexpressed with pan-B-cell markers, CD19 and CD20. Although the fluorescence intensity of HM1.24 was impaired by the fixation, all HM1.24<sup>+</sup> cells strongly expressed cytoplasmic IgM- $\lambda$ . These findings indicate that HM1.24 is also expressed by terminally differentiated B cells.

The anti-HM1.24 MoAb was found to be unreactive with most normal tissues, including peripheral blood, bone marrow, reactive lymphnodes, liver, spleen, kidney, and heart. Small numbers of positive cells in the section of bone marrow, lymph node, and spleen were identified as plasma cells based on morphologic features and the presence of cIg.

Sorting of HM1.24<sup>+</sup> cells from nucleated bone marrow cell populations obtained from a patient with rheumatoid arthritis showed that 99.6% of the isolated cells had mature plasma-cell morphology (Fig 2A) and contained cIg. HM1.24<sup>+</sup> marrow cells from a patient with IgG $\kappa$  myeloma had immature plasma-cell features (Fig 2B), and the cells were 100% cIg<sup>+</sup>. It was noted that the HM1.24<sup>+</sup> cells from another myeloma patient (IgG $\lambda$  type, at an advanced clinical stage) were composed of plasma and lymphocytoid cells (Fig 2C). These cells exhibited negative staining with the anti- $\kappa$  MoAb (Fig 2D) and positive (99.8%) with the anti- $\lambda$  MoAb (Fig 2E). Corresponding lymphocytoid cells were rarely seen among the HM1.24<sup>+</sup> cells from reactive marrow of the rheumatic patient.

**Induction of HM1.24 antigen on PWM-stimulated PBMC in vitro.** We found that PWM, which induces terminal differentiation of B cells in vitro,<sup>32</sup> also influenced HM1.24 antigen expression by normal B cells. Cells cultured in the presence of PWM were harvested daily, stained with FITC-anti-HM1.24 and PE-conjugated MoAbs (CD3, CD19, CD20, and CD38), and examined by flow cytometry (Fig 3). After 5 days of culture, a group of CD38<sup>+</sup>HM1.24<sup>+</sup> cells was evidenced and consisted of CD3<sup>+</sup> activated T cells. HM1.24<sup>+</sup> cells did not appear until day 10. Such cells were partly CD19<sup>+</sup> and CD38<sup>+</sup>, mostly CD20<sup>+</sup>, and clearly CD3<sup>-</sup> (data not shown). Cytoplasmic IgM or IgG was detected by immunoperoxidase staining of cells contained in the HM1.24<sup>+</sup> cytospin preparations (data not shown).

**Characterization of HM1.24 antigen.** Antibody binding studies showed that maximum binding levels of <sup>125</sup>I-anti-HM1.24 MoAb were higher on the plasma cell lines, RPMI 8226 and M1J, than on the IM-9 B-lymphoblastoid line (Fig 4A). These data verify the results of flow cytometric analyses. The affinity constant and the maximum number of binding sites for anti-HM1.24 were calculated from the binding curve using as reference standard the RPMI 8226 cells (Fig 4B). Competition experiments indicate no significant inhibition of the <sup>125</sup>I-anti-HM1.24 binding to RPMI 8226 cells by anti-CD38, anti-1<sup>CA</sup>-1, or MM4 MoAbs. In con-



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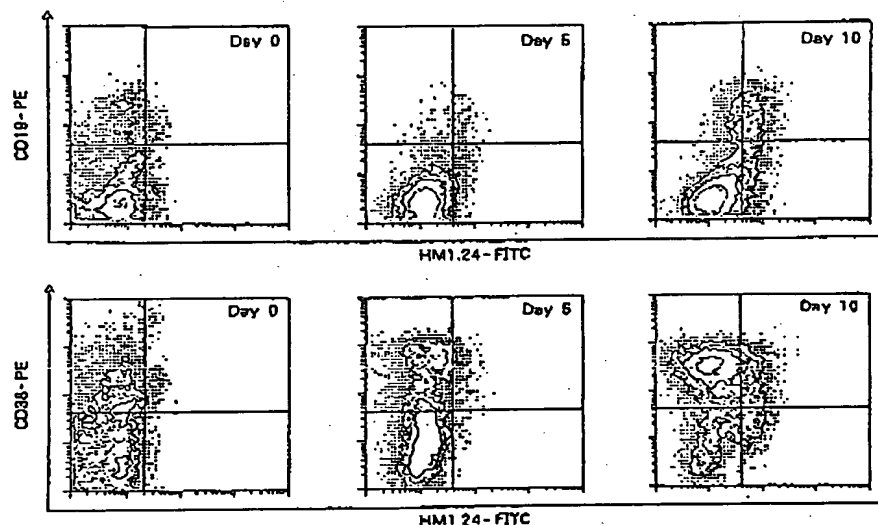


Fig 3. Flow cytometric analysis of HM1.24, CD19, and CD38 expression by PWM-stimulated normal peripheral mononuclear cells. In this assay, blocking of Fc receptors with  $\gamma$ -globulin was omitted not to disturb the subsequent Ig staining

trast, addition of the unlabeled anti-HM1.24 MoAb resulted in complete inhibition of binding (Fig 4C). Thus, the epitope identified by anti-HM1.24 on RPMI 8226 cells differed from those recognized by the other MoAbs.

Anti-HM1.24 MoAb specifically precipitated a diffuse band from  $^{125}$ I-surface-labeled RPMI 8226 proteins (Fig 5A). The band showed an apparent molecular weight (MW) of 29 to 33 kD under reduced conditions. Under unreduced conditions, the band migrated to higher MW of 56 to 62 kD.

## DISCUSSION

In this report, we have described the production and characterization of an MoAb reactive with the terminal B-cell-associated antigen HM1.24. To avoid complication caused by the presence of multilineage markers on fresh myeloma cells,<sup>23,25</sup> we selected a terminally differentiated B-cell line (KPC-32) that lacked undesired immunodominant determinants, such as HLA-DR, surface Igs, and EBV-associated antigens, for the immunization. Flow cytometry was used on intact cells to eliminate MoAbs reactive with cytoplasmic antigens expressed by plasma cells, eg, rough endoplasmic reticulum-associated antigens<sup>1</sup> and bcl-2 proto-oncogene product.<sup>16</sup>

In contrast to other plasma cell-associated antigens, CD38<sup>13-16</sup> and PCA-1,<sup>2</sup> the antigen recognized by the anti-HM1.24 MoAb and designated HM1.24 was not found on activated T cells or granulocytes. Additionally, cross-blocking experiments indicate that the epitope recognized by the anti-HM1.24 MoAb is distinct from those recognized by other anti-plasma-cell MoAbs, including anti-CD38, MM4, and anti-PCA-1. A human cell surface pan-epithelial glycoprotein encoded by the hEGP314 gene is expressed on plasma cell lines as well as on most epithelial cells and tumors.<sup>27</sup> Similar patterns of shared reactivity with epithelial tissue and plasma cells has also been observed on six plasma cell-reactive MoAbs raised against RPMI 8226 cells.<sup>28</sup> The

fact that anti-HM1.24 does not bind to various carcinoma lines, including WiDr, which strongly expresses the pan-epithelial glycoprotein, or to normal tissues indicates that HM1.24 is not an epithelial cell-associated antigen.

The results obtained by flow cytometric analyses on human cell lines tested, as well as on normal and neoplastic tissues and PWM-stimulated PBMC, are consistent with the lineage-specific expression of HM1.24 on terminally differentiated normal and neoplastic B cells. These immunophenotyping data suggest that the expression pattern of HM1.24 is distinct from known CD antigens on the differentiation pathway of normal B cells.<sup>29</sup> The terminal stage of B-cell differentiation is accompanied by the acquisition of clg and the secretion of Ig. Although most of the known phenotypic changes in this maturation process involve loss of various membrane antigens, new membrane proteins can be expressed. Although the function of HM1.24 molecules remains unknown, the expression of this antigen occurs concomitantly with Ig secretion by terminally differentiated B cells, ie, lymphoplasmacytoid and plasma cells. Purification of the antigen using immunoaffinity chromatography is underway so that the molecules can be identified and characterized further.

Immunoprecipitation of  $^{125}$ I-labeled surface proteins with the anti-HM1.24 MoAb identified a diffuse band by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 29 to 33 kD under reducing conditions. The presence of a higher MW band (56 to 62 kD) in the unreduced sample would suggest that the HM1.24 protein forms a disulfide-linked homodimer. Alternatively, the diffuse band may represent nonidentical subunits of a complex protein. Most likely, the band consists of a group of identical proteins with different glycosylation patterns. In these respects, this phenotypic marker differs from other characterized leukocyte-associated antigens. The major histocompatibility complex (MHC)-class II antigens represent glycoproteins of sim-

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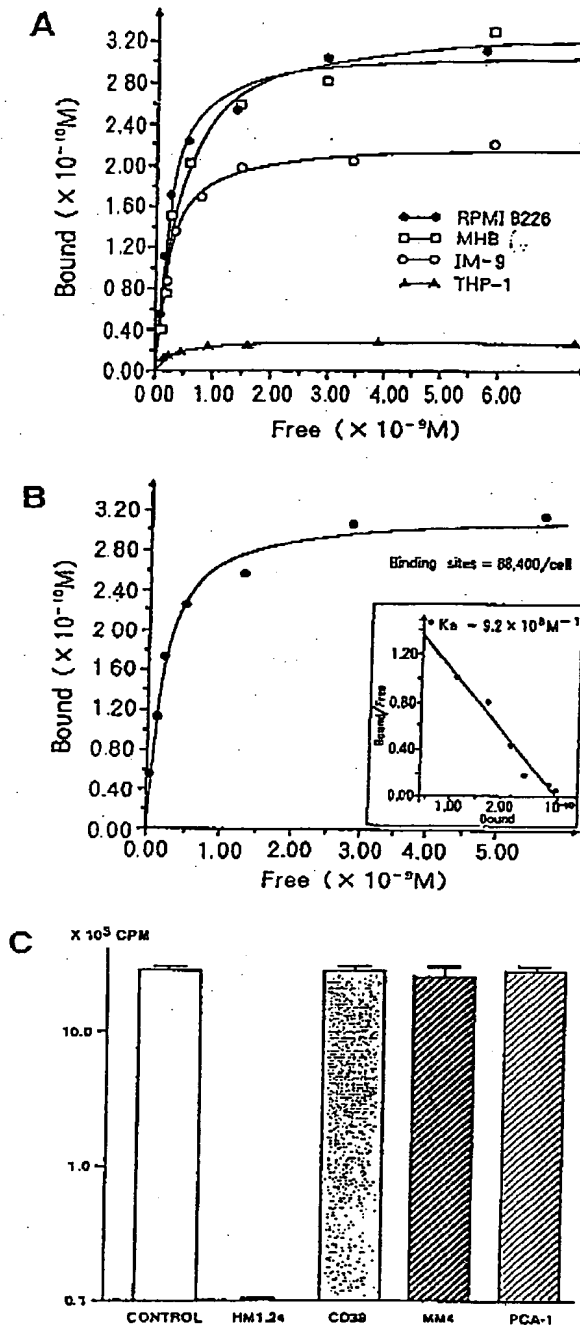


Fig 4. (A) Binding curves of  $^{125}$ I-labeled anti-HM1.24 MoAb to various cell lines. (B) Binding characteristic of  $^{125}$ I-labeled anti-HM1.24 MoAb to RPMI 8226 cells. Scatchard plot is shown in a small square. (C) Cross-blocking with unlabeled MoAbs, mouse IgG<sub>2a</sub> (control), anti-HM1.24, anti-CD38, MM4, and anti-PCA-1 of  $^{125}$ I-labeled anti-HM1.24 binding to RPMI 8226 cells.

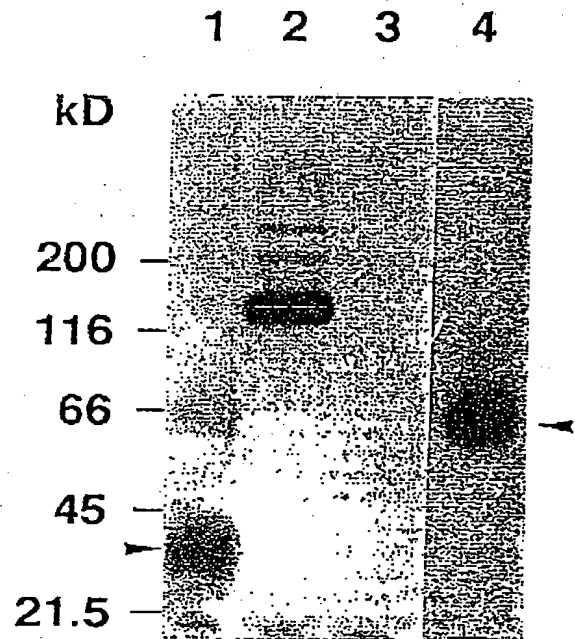


Fig 5. Autoradiography of immunoprecipitates with anti-HM1.24 (lane 1, reduced; and lane 4, unreduced), anti-HM1.43 (another plasma-cell-reactive MoAb, lane 2, reduced), and control mouse IgG<sub>2a</sub> (lane 3, reduced), from  $^{125}$ I-labeled lysates of RPMI 8226 cells. Arrowheads indicate the specific precipitin bands with anti-HM1.24 MoAb. Internal molecular weight markers (Bio-Rad) included myosin (200 kD), *Escherichia coli*  $\beta$ -galactosidase (116 kD), BSA (66 kD), hen egg white ovalbumin (45 kD), and soybean trypsin inhibitor (21.5 kD).

ilar MW to that of HM1.24 but, in contrast to this epitope, are expressed on many cell types.

The results of cell-sorting experiments support the concept that HM1.24<sup>+</sup> cells represent mature Ig-secreting cells. We have also identified HM1.24<sup>+</sup> lymphocytoid/lymphoplasmacytoid cells in the bone marrow of patients with multiple myeloma or Waldenström's macroglobulinemia. These cells presumably represent earlier forms of the malignant plasma cells.<sup>39,43</sup> The clonal involvement of early stage B cells in myeloma has been evidenced by the presence of pre-switch B cells bearing clone-specific Ig variable-region transcripts.<sup>11,44</sup> Because anti-HM1.24 MoAb does not react with early stage B cells, the narrow reactivity spectrum of this MoAb may limit its usefulness as a single reagent for immunotherapy of myeloma.<sup>43</sup> However, because of the high-affinity constant ( $\sim 10^9 M^{-1}$ ) of this MoAb, its IgG<sub>2a</sub> subclass, and the fact that it does not inhibit the growth of normal CFUs in vitro (unpublished observation), this reagent could be used to purge malignant cells from the bone marrow of patients with multiple myeloma in combination with other B-lineage-specific MoAbs.<sup>46-49</sup> Furthermore, the ability of anti-HM1.24 MoAb to separate by sorting malignant terminal B cells from earlier progenitor cells<sup>39-41</sup> can help define more precisely the initial stages of malignant B-cell transformation and identify factors that promote clonal evolution.

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In conclusion, we have described an anti-plasma-cell MoAb that defines a novel B-lineage-restricted antigen (HM1.24) selectively expressed on the surface of terminally differentiated normal and neoplastic B cells. The unique specificity of the anti-HM1.24 MoAb for lymphoplasmacytoid and mature plasma cells makes this reagent especially useful to elucidate the pathogenesis of multiple myeloma-related immunoproliferative disorders and to treat patients with these presently incurable diseases.

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## REFERENCES

1. Ling NR, MacLennan ICM, Mason DY: B-cell and plasma cell antigens: New and previously defined clusters, in McMichael AJ, Beverley PCL, Cobbold S, Crumpton MJ, Glicks W, Gough FM, Hogg N, Horton M, Ling N, MacLennan ICM, Mason DY, Milstein C, Spiegelhalter D, Waldmann H (eds): *Leucocyte Typing III*. Oxford, UK, Oxford, 1986, p 302
2. Anderson KC, Park EK, Bates MP, Leonard RCP, Hardy R, Schlossman SF, Nadler LM: Antigens on human plasma cells identified by monoclonal antibodies. *J Immunol* 130:1152, 1983
3. Anderson KC, Bates MP, Slaughenhaupt B, Schlossman SF, Nadler LM: A monoclonal antibody with reactivity restricted to normal and neoplastic plasma cells. *J Immunol* 132:3172, 1984
4. Tong AW, Lee JC, Stone MJ: Characterization of a monoclonal antibody having selective reactivity with normal and neoplastic plasma cells. *Blood* 69:238, 1987
5. Epstein J, Xiao H, He X-Y: Markers of multiple hematopoietic cell lineages in multiple myeloma. *N Engl J Med* 322:664, 1990
6. Terstappen LWMM, Johnsen S, Segers-Nolten IMJ, Loken MR: Identification and characterization of plasma cells in normal human bone marrow by high-resolution flow cytometry. *Blood* 76:1739, 1990
7. Loo R, Boeker M, Peest D, Hein R, Bartl R, Gessner JE, Seibach J, Wacker G, Deiner H: Multiparameter analysis of normal and malignant human plasma cells: CD38<sup>++</sup>, CD56<sup>+</sup>, CD54<sup>+</sup>, cIg<sup>+</sup> is the common phenotype of myeloma cells. *Ann Hematol* 64:132, 1992
8. Shimazaki C, Gotoh H, Ashihara E, Oku N, Inaba T, Murakami S, Itoh K, Ura Y, Nakagawa M, Fujita N: Immunophenotype and DNA content of myeloma cells in primary plasma cell leukemia. *Am J Hematol* 39:159, 1992
9. Hata H, Xiao H, Petrucci MT, Woodliff J, Chang R, Epstein J: Interleukin-6 gene expression in multiple myeloma: A characteristic of immature tumor cells. *Blood* 81:3357, 1993
10. Harada H, Kawano MM, Huang N, Harada Y, Iwato K, Tanabe O, Tanaka H, Sakai A, Kuramoto A: Phenotypic difference of normal plasma cells from mature myeloma cells. *Blood* 81:2658, 1993
11. Billadeau D, Ahmann G, Grcipp P, Van Ness B: The bone marrow of multiple myeloma patients contains B cell populations at different stages of differentiation that are clonally related to the malignant plasma cell. *J Exp Med* 178:1023, 1993
12. Alessio M, Roggero S, Funaro A, De Monte LB, Peruzzi L, Geuna M, Majevski F: CD58 molecule: Structural and biochemical analysis on human T lymphocytes, thymocytes and plasma cells. *J Immunol* 145:878, 1990
13. Tedder TF, Clement LT, Cooper MD: Discontinuous expression of a membrane antigen (HB-7) during B lymphocyte differentiation. *Tissue Antigens* 24:140, 1984
14. Terstappen LWMM, Hollander Z, Meiners H, Loken MR: Quantitative comparison of myeloid antigens on five lineages of mature peripheral blood cells. *J Leukoc Biol* 48:138, 1990
15. Janossay G, Tidman N, Papageorgiou ES, Kung PC, Goldstein G: Distribution of T lymphocyte subsets in the human bone marrow and thymus: An analysis with monoclonal antibodies. *J Immunol* 126:1608, 1981
16. Reinherz EL, Kung PC, Goldstein G, Levy RH, Schlossman SF: Discrete stages of human intrathymic differentiation: Analysis of normal thymocytes and leukemic lymphoblasts of T-cell lineage. *Proc Natl Acad Sci USA* 77:1588, 1980
17. Terstappen LWMM, Huang S, Safford M, Lansdorp PM, Loken MR: Sequential generations of hematopoietic colonies derived from single nonlineage-committed CD34<sup>+</sup>CD38<sup>+</sup> progenitor cells. *Blood* 77:1218, 1991
18. Goto T, Takishita M, Wada M, Tsuchihashi N, Miyamoto Y, Jo Z, Okagawa K, Kosaka M, Saito S: Establishment of a human myeloma cell line (KPC-32) and production of monoclonal antibodies to its surface antigen. *Jpn J Clin Hematol* 32:1400, 1991 (abstr)
19. Lozzio CB, Solomon A, Lozzio BB, Klepper MB: Characterization of a multiple myeloma cell line of human origin. *IRCS Med Sci* 3:10, 1975
20. Goto T, Abe M, Kosaka M: A new immunization protocol for the production of monoclonal antibodies specific for VL subgroups of human immunoglobulins. *Tokushima J Exp Med* 37:89, 1990
21. de St Groth SF, Scheidegger D: Production of monoclonal antibodies: Strategy and tactics. *J Immunol Methods* 35:1, 1980
22. Kennel SJ, Foote LJ, Lankford PK: Analysis of surface proteins of mouse lung carcinomas using monoclonal antibodies. *Cancer Res* 41:3465, 1981
23. Pusner MR, Antoniou D, Griffin J, Schlossman SF, Lazarus H: An enzyme-linked immunosorbent assay (ELISA) for the detection of monoclonal antibodies to cell surface antigens on viable cells. *J Immunol Methods* 48:23, 1982
24. Abe M, Goto T, Kennel SJ, Wolfenbarger D, Macy SD, Weiss DT, Solomon A: Production and immunodiagnostic applications of anti-human light chain monoclonal antibodies. *Am J Clin Pathol* 100:67, 1993
25. Solomon A, Weiss DT, Macy SD, Antounouel RA: Immunocytochemical detection of kappa and lambda light chain V region subgroup in human B-cell malignancies. *Am J Pathol* 137:855, 1990
26. Kennel SJ, Chen JP, Lankford RK, Foote LJ: Monoclonal antibodies from rats immunized with fragment of D of human fibrinogen. *Thrombosis Res* 22:309, 1981
27. Kennel SJ: Characterization of a tumor cell surface protein with heterologous antisera to a spontaneous BALB/c lung carcinoma. *Cancer Res* 39:2934, 1979
28. Kennel SJ, Foote LJ, Falcioni R, Sonnenberg A, Stringer CD, Crouse C, Helmer ME: Analysis of the tumor-associated antigen 7SP-180. *J Biol Chem* 264:15515, 1989
29. Anderson KC, Bates MP, Slaughenhaupt BL, Pinkus GS, Schlossman SF, Nadler LM: Expression of human B cell-associated antigens on leukemias and lymphomas: A model of human B cell differentiation. *Blood* 63:1424, 1984
30. Jensen GS, Andrew EJ, Munt MJ, Vergidis R, Ledbetter JA, Pilarski LM: Transitions in CD45 isoform expression indicate continuous differentiation of a monoclonal CD5<sup>+</sup>CD11b<sup>+</sup> B lineage in Waldenström's macroglobulinemia. *Am J Hematol* 37:20, 1991
31. Kiyokawa N, Kokai Y, Ishimoto K, Fujita H, Fujimoto J, Hata J-I: Characterization of the common acute lymphoblastic leukemia antigen (CD10) as an activation molecule on mature human B cells. *Clin Exp Immunol* 79:322, 1990

32. Stashenko P, Nadler LM, Hardy R, Schlossman SP: Expression of cell surface markers after human B lymphocyte activation. *Proc Natl Acad Sci USA* 78:3848, 1981
33. Warburton P, Joshua DE, Gibson J, Brown RD: CD10-(CALLA)-positive lymphocytes in myeloma: Evidence that they are a malignant precursor population and are of germinal centre origin. *Leuk Lymphoma* 1:11, 1989
34. Van Camp B, Durie BGM, Spier C, De Wacle M, Van Riet I, Vela E, Frutiger Y, Richter L, Orlan TM: Plasma cells in multiple myeloma express a natural killer cell-associated antigen: CD56 (NKH-1; Leu-19). *Blood* 76:377, 1990
35. Jensen GS, Belch AR, Mant MJ, Ruetter BA, Pilarski LM: Expression of multiple adhesion molecules on circulating monoclonal B cells in myeloma, in Porter M, Melchers F (eds): *Current Topics in Microbiology and Immunology*, vol 182. Berlin, Germany: Springer-Verlag, 1992, p 187
36. Hamilton MS, Barker HF, Ball J, Drew M, Abbot SD, Franklin IM: Normal and neoplastic human plasma cells express bcl-2 antigen. *Leukemia* 5:768, 1991
37. Bergsagel PL, Victor-Kobrin C, Timblin CR, Trepel J, Koehl WM: A murine cDNA encodes a pan-epithelial glycoprotein that is also expressed on plasma cells. *J Immunol* 148:590, 1992
38. Johnson GD, Ling NR, Nathan PD, Hardie DL: Use of monoclonal antibodies reactive with secretory epithelial cells for the immunocytochemical identification of plasma cells. *Immunol Lett* 12:295, 1986
39. Ruiz-Argüelles GJ, Katzmann JA, Grelpp PR, Gonchoroff NJ, Garton JP, Kyle RA: Multiple myeloma: Circulating lymphocytes that express plasma cell antigens. *Blood* 64:352, 1984
40. Epstein I, Barlogie B, Katzmann J, Alexanian R: Phenotypic heterogeneity in aneuploid multiple myeloma indicates pre-B cell involvement. *Blood* 71:861, 1988
41. King MA, Nelson DS: Tumor cell heterogeneity in multiple myeloma: Antigenic, morphologic, and functional studies of cells from blood and bone marrow. *Blood* 73:1925, 1989
42. Omedé P, Boccadoro M, Gallone G, Frieri R, Battaglio S, Reddoglia V, Pileri A: Multiple myeloma: Increased circulating lymphocytes carrying plasma cell-associated antigens as an indicator of poor survival. *Blood* 76:1375, 1990
43. Jensen GS, Mant MJ, Belch AJ, Berenson JR, Ruetter BA, Pilarski LM: Selective expression of CD45 isoforms defines CALLA<sup>+</sup> monoclonal B-lineage cells in peripheral blood from myeloma patients as late stage B cells. *Blood* 78:711, 1991
44. Corradini P, Boccadoro M, Voena C, Pileri A: Evidence for a bone marrow B cell transcribing malignant plasma cell VDJ joined to C $\mu$  sequence in immunoglobulin (IgG)- and IgA-secreting multiple myelomas. *J Exp Med* 178:1091, 1993
45. Chan CSP, Wormsley SB, Pierce LE, Peter JB, Schechter GP: B-cell surface phenotypes of proliferating myeloma cells: Target antigens for immunotherapy. *Am J Hematol* 33:101, 1990
46. Tong AW, Lee J, Wang R-M, Dalton WS, Tsuruo T, Fay JW, Stone MJ: Elimination of chemoresistant multiple myeloma clonogenic colony-forming cells by combined treatment with a plasma cell-reactive monoclonal antibody and a P-glycoprotein-reactive monoclonal antibody. *Cancer Res* 49:4829, 1989
47. Lemoli RM, Gobbi M, Tazzari PL, Tassi C, Dinota A, Visani G, Grassi G, Mazza P, Cavo M, Tura S: Bone marrow purging for multiple myeloma by avidin-biotin immunoadsorption. *Transplantation* 47:385, 1989
48. Shimazaki C, Wisniewski D, Scheinberg DA, Atzpodien J, Strife A, Gulati S, Fried I, Wisniewski R, Wang CY, Clarkson BD: Elimination of myeloma cells from bone marrow by using monoclonal antibodies and magnetic immunobeads. *Blood* 72:1248, 1988
49. Anderson KC, Barut BA, Ritz J, Freedman AS, Takvorian T, Rabinowe SN, Soiffer R, Heflin L, Coral F, Dear K, Mauch P, Nadler LM: Monoclonal antibody-purged autologous bone marrow transplantation therapy for multiple myeloma. *Blood* 77:712, 1991